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(54) Title: DNA-CLONING METHOD USING A CRE-LOX VECTOR UNDER CONDITIONS OF MACROMOLECULAR CROWDING

(57) Abstract

The method uses a plasmid vector, p91ox5, containing a site-specific recombination sequence lox from the lox/Cre recombinase system of bacteriophage P1. There are two distinct stages. Firstly, vector and fragment DNAs are ligated intermolecularly under conditions of macromolecular crowding (15% polyethylene glycol 6000). Secondly, circular recombinant molecules are excised from the ligation products by Cre recombinase acting on pairs of lox sites within directly repeated vector molecules flanking insert DNA. Recombinants are introduced into cells conventionally by transformation or electroporation. Applications of the technique to cDNA library generation and recovery of DNA from archive material are discussed.

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DNA-CLONING METHOD USING A CRE-LOX VECTOR UNDER CONDITIONS OF MACROMOLECULAR CROWDING.

## Field of the Invention

This invention relates to a rapid method of cloning a DNA molecule, particularly a blunt-ended DNA molecule, and to a novel plasmid of use in performing the method.

## Background to the Invention

The ligation of blunt-ended DNA fragments is much less efficient than that of cohesive-ended fragments under normal reaction conditions. The quantitative cloning of such fragments (produced for example by cDNA synthesis or PCR amplification) requires elaborate and time-consuming techniques. Long incubation times and high concentrations of ligase and DNA are required to promote the reaction by conventional means (1). Alternative methods involve the addition of linkers to fragment ends rendering them cohesive and thus easily clonable (2).

It has been known for about a decade that the efficiency of ligation in general, and that of blunt ends in particular, can be dramatically increased in conditions of macromolecular crowding induced by the presence of high concentrations of varius inert polymers (3-5). All such joining, however, is intermolecular, so these conditions cannot be used directly for the creation of plasmid

recombinants. A technique has been described (6) in which the concatemers generated by ligation in 15% polyethlyene glycol (PEG) are cleaved by a vector-unique restriction enzyme and then ligated under PEG-free conditions to produce the required circular molecules.

This application concerns a similar but more straightforward two-stage technique in which the second stage restriction/ligation steps of the published method (6) are replaced by a single, rapid site-specific recombination mediated by the lox/Cre system of bacteriophage P1(7). [Cre is a recombinase which acts specifically on the 34bp lox sequence: its natural function is to circularize P1 genomes which when injected into cells are linear 100kb molecules flanked by lox sites in direct repeat (8). Unlike many recombinases, Cre is not fastidious and works efficiently in vitro under conditions similar to those used for restriction enzyme digestion (9).] The principle of the second stage has been outlined previously (10) and proposed as a means of cloning large fragments into plasmids. However, the addition here of the highly efficient ligation step allows this principle to be exploited in a more general technique for plasmid cloning. The whole process, designated 'turbo cloning', is quick (1 - 2h), simple and efficient.

Another system which uses PEG to promote ligation has been published (11). In the presence of 10% PEG, the rate of blunt end ligation is considerably enhanced, but intramolecular ligation is not abolished. (For simplicity, this procedure will be referred to hereafter as the 10% PEG method).

Summary of the Invention

In one aspect, the present invention provides a method of cloning a DNA molecule, comprising:

a) ligating a linear insert DNA molecule to linear vector DNA molecules, which vector molecules contain a single lox site, under conditions of macromolecular crowding so as to form at least some suitably ligated DNA in which a linear insert DNA molecule is joined at each end to a respective molecule of linear vector DNA wherein the two linear vector DNA molecules immediately adjacent to the insert DNA have the relationship of being direct repeats;

b) bringing the products of the ligation reaction, no longer under conditions of macromolecular crowding, into contact with Cre protein so as to cause circularisation of the suitably ligated DNA; and

c) dissociating the Cre protein from the DNA.

Step b) may be achieved intracellularly by introducing the DNA into a cell which is capable of expressing Cre, preferably using a cell which is capable of inducible expression of Cre.

Alternatively, step b) may be achieved extracellularly, in which case the method further comprises as step d) introducing the DNA into a suitable host.

The Cre protein is conveniently dissociated from the DNA by heating.

Conditions of macromolecular crowding can be achieved by the presence of 15% (w/v) polyethylene glycol 6000.

The conditions of macromolecular crowding are conveniently

removed by dilution of the reaction mixture.

The vector molecule is preferably p9lox5.

The vector has preferably been dephosphorylated prior to ligation.

The linear insert DNA may have blunt ends.

In a further aspect, the present invention provides the plasmid p9lox5 as herein defined.

When performing the invention, recombinant circles are generated directly as in PEG-free conditions, but the time taken to perform the reaction can be reduced from 16 h to 30 min.

The invention is further illustrated in the following description of model cloning experiments which compare the present invention with the prior art. Reference is made to the accompanying figures, in which:

Figure 1 shows results obtained on an agarose gel;

Figure 2 illustrates schematically the method of the present invention; and

Figure 3 shows further results obtained on an agarose gel.

MATERIALS AND METHODS

Materials

DNAs were prepared by standard methods (1) and restriction enzymes used according to the supplier's (Boehringer) recommendations.

Construction of p9lox5

A SalI cohesive ended duplex containing lox was made by annealing the 40-mers B16 (5'-TCGACATAACTTCGTATAATGTATGCTATACGAAGTTATG-3') and B17 (5'TCGACATAACTTCGTATAGCATACATTATACGAAGTTATG-3') synthesized using an Applied Biosystems DNA Synthesizer Model 381A. A single copy of the duplex was cloned into SalI-cleaved pUC9(12) to generate p9lox5 whose structure was confirmed by sequencing: the orientation is such that B16 is inserted into the lacZ' sense strand, with the consequential loss of the blue/white test for recombinants. This 2705bp vector retains the unique polylinker SmaI site of pUC9.

Preparation of p9lox5 for cloning

It is convenient to prepare stocks of linearized, dephosphorylated vector. Microgram quantities of plasmid purified from ethidium bromide/CsCl gradients were cleaved with SmaI and dephosphorylated by calf intestinal phosphatase (Promega) using standard techniques (1). To reduce the background caused by traces of uncut plasmid, linear DNA was purified by excision from an agarose gel and extracted using a freeze-squeeze method (13) followed by desalting in G-50 (Sephadex, Pharmacia) spin columns.

Concentration of vector was determined by estimating the band density of aliquots run on agarose gels.

Turbo cloning

This is a two stage process. In stage one, the vector and fragment DNAs (in water or TE: 10mM Tris.HCl(pH 8.0), 1mM EDTA) are combined in a ligation buffer similar to that described (4) (final concentrations: 50mM Tris.HCl (pH 8.0), 0.5mM ATP, 0.5mM dithioerythritol (DTE), 5mM $MgCl_2$) and an appropriate volume of PEG 6000 (BDH) added from a filter-sterilized 40% (w/v) stock solution to give a final concentration of 15%. Lastly, 0.3 to 0.5 units of T4 DNA ligase (Boehringer) is added giving a total reaction volume of 10 - 25ul. The reaction is mixed carefully and thoroughly by pumping through a micropipettor tip before incubation at room temperature for 10 - 30 min. The reaction is terminated by heat inactivation of ligase at 75° for 10 - 15 min.

The second stage is initiated by adding four volumes of Cre buffer (10mM Tris.HCl(pH7.5), 10mM $MgCl_2$, 50mM NaCl, 1mM DTE) containing 0.15ug of Cre protein (NEN). The reaction is incubated at 30° for 20 - 30 min before heat inactivation as before. (This is essential: unlike restriction enzymes and ligase, Cre remains bound to DNA and if not heat-dissociated interferes with cell uptake). The process is now complete and the DNA may be introduced into cells by chemical transformation or electroporation as usual. In this work, electrocompetent E. coli strain DH10B(14) was used. Desalting was achieved by 15min drop dialyses of small aliquots of reaction mix on nitrocellulose filters (Millipore type VM, 0.05u pore size) floating on water (15). 1-2ul of the dialysate was

electroporated into 30 - 35ul of electrocompetent cells (Bio-Rad Gene Pulser: 2.5kV, 25uF, 200 ohm). Subsequent steps were as described (14), except that all media used were L broth based.

Some general points must be noted. (i) Accurate pipetting is essential in stage one as even quite small deviations from 15% PEG can significantly reduce the macromolecular crowding effect (4): it helps to thaw the 40% stock solution to room temperature at which it is less viscous. (ii) PEG may be removed from the stage one products by chloroform extraction or DNA precipitation (4), but dilution is quicker and minimizes DNA loss. (iii) Vector dephosphorylation is advised for two reasons: firstly, the background of regenerated vector is reduced; secondly, the long concatemers with multiple lox sites otherwise produced may be converted by Cre into complex knotted structures that could sequester the desired recombinant circles (although this has not been investigated).

Preparation of Alu-PCR products for ligation

Total genomic DNA from a hybrid cell lines B2.13 derived from J1 (clone 4) (16) which essentially contains human chromosome 11 in a CHO background was a gift from Vivienne Watson. An oligonucleotide, 614, (5'-GTGAGCCGAGATCGCGCCACTGCACT-3') designed from the consensus Alu repeat sequence (17) was used to prime amplification of inter-Alu regions in this DNA. Promega enzyme and buffer were used with a standard program on a Hybaid Omnigene thermal cycler. Samples were run on a 1.8% agarose gel (Fig.1) revealing an array of bands in the size range 0.5 - 2.5kb. An aliquot containing ca. 0.5ug was diluted five-fold in buffer and treated with T4

DNA polymerase (Boehringer) with an excess of dNTPs to render all fragments uniformly blunt-ended (18). After heat inactivation of the reaction (in the presence of 20mM EDTA), primers, dNTPs, salts and most short (less than 500bp) DNA fragments were removed by Geneclean (Bio 101) extraction and the DNA eluted into water. The blunt ended fragments were them phosphorylated by polynucleotide kinase (Boehringer) as described (18). The enzyme was heat inactivated and a 15 min drop dialysis step (see above) was used to remove salts and the DNA concentration estimated by running an aliquot on a gel.

RESULTS

Principle of the method

(See Fig. 2). As in the method described (6), there are two stages: (i) ligation enhanced by macromolecular crowding and (ii) circularization in non-crowding conditions.

The first stage is common to both methods. For turbo cloning, a plasmid vector containing a lox site must be used: linearized, blunt-ended vector (p91ox5) and fragment DNAs are ligated in 15% PEG at room temperature. Under these conditions, blunt end ligation is almost as efficient as cohesive end ligation (4), and essentially all phosphorylated termini are joined in a short time (less than 1h) by moderate amounts of T4 DNA ligase. To maximise recovery of distinct recombinants, there must be a large molar excess of vector so that most insert DNA fragments become flanked by vector molecules. (Note that the intermolecular nature of the joining is a distinct advantage since it prevents the self-circularization which

sequesters insert fragments. The end products of this stage are long concatemers of vector and fragment DNA unless the vector is dephosphorylated, in which case fragment DNA (if present as a small proportion) will almost quantitatively be converted to linear vector::fragment::vector trimers. Half of these hybrid trimers will have the vector components (and hence lox sites) in inverted repeat, and half in direct repeat.

In stage 2, the reaction mix is diluted to abolish macromolecular crowding and Cre protein added. The 50% of hybrids with lox in direct repeat will be productively resolved to the desired circular recombinants (plus vector monomers). The other hybrids will undergo unproductive rounds of inversion between lox sites and remain linear. Thus turbo cloning is subject to a theoretical maximum efficiency of 50%. (Although Cre works in the opposite direction of integrating circles into linears to regenerate linear hybrids, the equilibrium is strongly in favour of circle formation (19).) After heat inactivation of Cre, stage 2 is complete and the DNA can be introduced into cells.

In the absence of macromolecular crowding, where both inter- and intramolecular ligation is permitted (as in conventional cloning and the 10% PEG method (11)), the yield of circular recombinants is heavily dependent both on the relative concentrations of vector and fragment DNAs and the total DNA concentration (20). Deviation from optimum concentrations can significantly reduce the yield! This makes the efficient cloning of low, indeterminate amounts of blunt-ended DNA especially difficult. The two-stage processess described here and in (6) are less dependent on total DNA concentration: in 15% PEG, it

appears that the effective concentration of abutting ends is so high (over a concentration range of at least 0.5 - 50ug/ml) that ligation rate depends almost exclusively on enzyme concentration (4). Furthermore, so long as vector DNA is in large molar excess (10:1 or greater), cloning should be quantitative even if the amount of fragment DNA is not known exactly.

The mathematical anaylsis of the effects of varying the vector/fragment ratio on product formation in conditions of macromolecular crowding is greatly simplified by the absence of intramolecular events and will be presented elsewhere.

Blunt-end cloning of the chloramphenicol acetyltransferase (CAT) gene into p91ox5.

A plasmid containing a selectable marker was chosen as the source of insert fragment for convenient identification of recombinants. The 4.3kb plasmid pACB104 replicates via the lambda dv replicon and carries the CAT gene encoding resistance to chloramphenicol (21): it was linearized at the unique EcoRV site within the O gene generating a blunt-ended fragmemt. After ligation to SmaI-linearized, dephosphorylated p91ox5 vector in a turbo cloning reaction, aliquots of reaction mix were electroporated into DH10B cells which had an electrocompetence level of 1 - 2 x $10^8$ cfu/ug pUC19. Cells were plated out on L agar containing ampicillin (Ap) and chloramphenicol (Cm) to select recombinants directly. Parallel experiments in which identical amounts of DNA were ligated under conditions described for the 10% PEG method (11) were also carried out (Table 1).

These results show that turbo cloning of blunt-ended fragments produces recombinants at least 40 times as efficiently as the 10% PEG method (11) over this range of DNA concentrations. Yields are satisfactory even when the concentration of fragment DNA in the stage 1 ligation mix is as low as 0.1ug/ml. Other turbo cloning experiments with these substrate DNAs, some using chemically competent cells, gave yields comparable to the above. Control experiments in which either PEG or Cre were omitted from the procedure produced at least a hundredfold fewer colonies.

The structures of a number of plasmids isolated from $Ap^r$ $Cm^r$ colonies were determined by restriction analysis: most were 7kb recombinant plasmids with single insertions of the 4.3kb fragment at the vector SmaI site. Interestingly, all had the insert in the same orientation, i.e., with CAT gene transcription opposing transcription from the vector lacZ' promoter. It must be assumed that recombinants with inserts in the other orientation are inviable in DH10B. This model system therefore underestimates the yield possible for turbo cloning a blunt-ended 4.3kb fragment by about 50%. Plasmid DNA from one clone, designated p9lox5Cm, was purified by banding in an ethidium bromide/CsCl gradient.

Comparison of turbo cloning of blunt-ended and cohesive-ended fragments

By linearizing both p9lox5 and pACB104 with EcoRI (which cuts the latter plasmid within the lacZ' gene (21)), the cohesive end analogue of the above cloning experiment could be carried out. Parallel turbo cloning experiments using either blunt-ended or cohesive-ended substrates with

vector:fragment ratios of 10:1 were therefore performed and recombinants selected as before (Table 2).

It is clear that the turbo cloning efficiencies of blunt-ended and cohesive-ended fragments obtained (i) are comparable to each other (bearing in mind the underrepresentation of SmaI/EcoRV-mediated hybrids discussed above) and (ii) represent a satisfactorily high proportion of the yield that would result if every fragment molecule was converted to a recombinant 7kb circle (i.e. as mimicked by the dummy reaction containing uncut p9lox5Cm).

Turbo cloning of Alu-PCR products

Approximately 10ng of blunt-ended, kinased Alu-PCR product DNA (see Materials and Methods) was ligated to 200ng of SmaI-linearized, dephosphorylated p9lox5 vector by turbo cloning. (This represents an approximate 10:1 vector molar excess, since the mean size of Alu-PCR product is 1.3 - 1.5kb (Fig. 1)) DH10B cells of the same electrocompetence as before were electroporated with aliquots of the ligated DNA and plated out on L agar containing ampicillin. About $10^7$ $Ap^r$ colonies per ug Alu-PCR DNA were obtained. Plasmids from 36 clones were examined both by Alu-PCR and restriction analysis: six had Alu-PCR fragments (ranging in size from 0.2 to 2kb) inserted (Fig. 3), the rest, reconstituted vectors, are presumed to have arisen from incomplete SmaI digestion or dephosphorylation of p9lox5. The yield of recombinants is therefore about $2 \times 10^6$ per ug fragment DNA. Given that the Alu-PCR fragments had to be blunt ended and phosphorylated prior to cloning, this yeld of recombinants is gratifyingly high. Restriction mapping of the

recombinants (data not shown) proved them to be unrelated. Thus, turbo cloning permits the efficient recovery of PCR products with minimal preprocessing and low selectivity.

DISCUSSION

Both in a model system and a practical application, turbo cloning produces recombinants from blunt-ended fragments at levels comparable to those obtained previously (6). (As seen above, the method may also be used for the cloning of cohesive-ended fragments, but the increase in efficiency is not so great. Nevertheless, as in the 10% PEG method (11), the saving in time may make it an attractive method to use for both kinds of cloning). The ease with which PCR products can be cloned is particularly notable, since many other methods rely on including restriction sites at the 5' end of primers to permit cohesive-end cloning of products. With turbo cloning, there is no need for such considerations in primer design.

The disadvantages of (6) are (i) the time-consuming restriction and ligation steps of stage 2 and (ii) the potential problem of cloning inserts which contain sites for the second stage restriction enzyme. The use of a rare cutter like NotI may substantially reduce (ii): turbo cloning does not have this problem because lox sites, being 34bp long, are unlikely to occur at all in most genomes. However, the new method, unlike others, requires specialized plasmid vectors carrying lox. A more versatile plasmid than p9lox5 derived from BlueScribe (22) and containing lox outwith the alpha-complementing lacZ' gene fragment (thus allowing the blue/white screen for recombinants) has now also been constructed for this

purpose.

In conventional plasmid cloning, for kinetic reasons, there is a bias towards the insertion of smaller fragments from a heterogenous population of DNA (20): the cloning of larger fragments therefore usually requires a prior size fractionation. Because of the intermolecular nature of joining in 15% PEG, size selection here should not occur. (It must be remembered, however, that all plasmid cloning methods are confronted by an obligatory size selection at the cell uptake stage: the probability that a plasmid will transform a competent cell is inversely proportional to its size (23).)

As well as the cloning of PCR products, turbo cloning could usefully be applied (as with (6)) to the generation of cDNA plasmid libraries. Blunt-ended cDNAs could be cloned directly and efficiently, with many fewer steps. However, this would require the use of maximally electrocompetent cells to compete with the efficiencies obtained with lambda expression vectors (1).

Another application of turbo cloning and similar methods may be the recovery of DNA from scarce archive material such as paraffin blocks, forensic samples and ancient biological specimens. The fraction of DNA surviving in an intact state in this material is usually tiny (24): because turbo cloning maximises the recovery of small amounts of blunt-ended DNA, and requires minimal preprocessing (making ends flush with T4 DNA polymerase may be all that is required), it could be more effective than existing approaches (25,26). PCR methods for examining such material, though extremely sensitive, suffer from size and sequence selectivity and can give

misleading results due to strand switching and misincorporation of bases during amplification.

Although yields obtained to date with turbo cloning are satisfactory, there seems little doubt that they could be improved by optimizing reaction conditions and/or vector structure. For example, the process might be speeded up by allowing the Cre recombination of stage 2 to take place in vivo, i.e., by introducing the first stage products directly into a strain constitutively expressing Cre protein. Preliminary experiments have shown that this is feasible, but unfortunately the viability of such strains carrying p9lox5 and recombinant derivatives is poor, possibly because of lox/Cre complexes interfering with plasmid replication or segregation. Use of an inducible strain that produces Cre only during DNA uptake would solve this problem, but no such strain has yet been described.

In vivo use of Cre to achieve circularisation

As outlined in the Discussion above, the Cre step of turbo cloning may be achieved in vivo. In one such experiment, 80ng of SmaI-cleaved p9lox5 was ligated to 100ng SmaI-cleaved pACB104 in the presence of 15% PEG 6000. (SmaI cleaves pACB104 to give two fragments of 2.4kb and 1.9kb, the latter containing the $Cm^r$ gene: thus this differs slightly from the model system used for the in vitro experiments in which linearized pACB104 was cloned in its entirety into p9lox5.) There is a slight molar excess of vector over 1.9kb fragment.

An aliquot of the ligation reaction was used to transform chemically competent cells of the strain NS3145 which

produces Cre constitutively from a prophage. Cells were plated out on L agar containing Ap and Cm to select recombinants. Colonies were obtained in numbers suggesting a blunt-ended cloning efficiency comparable to that achieved when Cre circularisation is performed in vitro (i.e., of the order of 1 - 10% of maximum possible efficiency: c.f. Table 2). A control experiment performed at the same time in which water was substituted for PEG in the ligation reaction yielded 40- to 50-fold fewer colonies, and no colonies were obtained when a $Cre^-$ strain (JM83) was used instead of NS3145. Thus both the use of PEG in the ligation reaction and the presence of Cre in the cells are necessary to achieve efficient cloning.

Some recombinant plasmids (after transfer to JM83 for ease of purification) were analysed by restriction digestion and shown to contain the vector and insert fragments, though complex chimaeras with multiple components were also observed. This is to be expected if, as in the experiment described here, the molar excess of vector over fragment is low.

Improved host/vector system for turbo cloning

As discussed in the paper (Boyd, Nucl. Acids Res. (1993), 21, 817-821), turbo cloning could be simplified further by carrying out the Cre circularisation step in vivo rather than in vitro, for example by use of a transiently induced Cre gene to confine Cre expression to the time at which linear concatemeric DNA from the PEG ligation step was transformed into cells. However, this could more simply be achieved with the use of a suitable host/vector system in which the host strain would contain a plasmid expressing Cre constitutively. The incoming turbo cloning vector would be designed to switch off replication of the resident plasmid, which would then rapidly be lost during colony formation. In more detail, the components of the system would be as follows:

* Host strain: an E. coli strain carrying a plasmid (with a different origin of replication and antibiotic resistance determinant from the turbo cloning vector) expressing the Cre protein. For example, this could be the lambda dv-based chloramphenicol resistance encoding plasmid pACB104 (Boyd et al. Mol. Gen. Genet., (1989), 217, 488-498) with the Cre gene cloned into the polylinker under control of the lac promoter. It may be desirable also to use a strain lacking RecBCD nuclease activity to minimise degradation of incoming linear DNA prior to Cre-mediated circularisation (Sauer & Henderson, Gene, (1988), 70,331-341).

Vector: a plasmid carrying a lox site and a trans-

acting incompatibility determinant specific for the Cre-expressing plasmid in the host strain. For example, with the lambda dv-based host plasmid described above, a suitable determinant would be the lambda cI or cro gene under control of a strong constitutive promoter. Such a vector could be made by cloning this promoter-cI/cro cassette into an existing turbo cloning vector such as p9lox5 described in the published work (Boyd, Nucl.Acids Res. (1993), 21, 817-821).

With a host/vector system configured as above, vector and insert DNA ligated in the presence of 15% PEG 6000 as in the standard protocol (Boyd, Nucl. Acids Res. (1993), 21, 817-821) would be transformed directly into competent cells of the host strain. Selection would then be applied for the incoming plasmid. Cre protein already in the cells produced by the resident plasmid would carry out the necessary circularisation of incoming DNA. The incompatibility determination on the incoming plasmid would then be expressed and abolish replication of the resident plasmid. By the time a colony was formed, no trace of the Cre expression plasmid would remain to complicate analysis of recombinants.

Table 1. Comparison of turbo cloning and 10% PEG[a] methods

| V:F ratio[b] | Ap Cm colonies per ug fragment | |
|---|---|---|
| | Turbo cloning[c] | 10% PEG[d] |
| 1.5:1 | $1.2 \times 10^6$ | $2.9 \times 10^4$ |
| 3:1 | $2.2 \times 10^6$ | $1.7 \times 10^4$ |
| 7:1 | $6.1 \times 10^5$ | $1.5 \times 10^4$ |
| 36:1 | $2.6 \times 10^5$ | $< 5 \times 10^3$ |

[a]Ref. (11)

[b] Molar ratio of vector (SmaI-cut p9lox5) to fragment (EcoRV-cut pACB104).

[c]The V:F ratios were achieved by ligating constant amounts of (30ng) of vector (using 0.3 units T4 DNA ligase - Boehringer) to varying amounts of fragment in a volume of 12ul for 20 min at room temperature (stage 1). Total DNA concentration varied from 2 to 7 ug/ml. The second stage Cre reaction was carried out in a volume of 60ul for 30 min at 30°.

[d]Ligations were carried out with the same amounts of DNA and ligase as above, but in 12ul of 10% PEG buffer (11) for 30 min at 16°.

Table 2. Relative efficiencies of blunt end and cohesive end turbo cloning

| Type of substrates | $Ap^r$ $Cm^r$ colonies per ug fragment | Efficiency index[b] (%) |
|---|---|---|
| Blunt ended | $1.2 \times 10^6$ | 3.8 |
| Cohesive ended | $4.0 \times 10^6$ | 12.8[c] |

[a] 100ng of SmaI or EcoRI-cut dephosphorylated vector (p9lox5) were ligated to 16ng EcoRV- or EcoRI-cut fragment (pACB104) in two turbo cloning reactions otherwise carried out as in footnote c of Table 1. In a dummy third reaction (omitting enzymes only), 26ng uncut p9lox5Cm (containing the equivalent of 16ng fragment) were processed similarly and gave rise to $3.2 \times 10^7$ colonies per ug fragment equivalent.

[b] Ratio of numbers of recombinant colonies obtained to those yielded by uncut p9lox5Cm.

[c] Both possible orientations of the EcoRI-mediated recombinants were found, although colonies bearing one of the forms grew poorly on subsequent purification.

FIGURE LEGENDS

Figure 1. Alu-PCR products of B2.13 DNA. DNA was amplified as in Materials and Methods, and 20ul aliquots of three independent 50ul reactions were run on a 1.8% agarose gel (lanes 2 - 4). Lane 1 contains size markers (1kb ladder, BRL: sizes shown in kb).

Figure 2. Overview of turbo cloning. (a) Stage 1: Linearized vector DNA ( ⟶ : arrow denotes lox site and its orientation) and insert DNA ( ——— ) are mixed in the presence of 15% PEG 6000 to produce conditions of macromolecular crowding. (b) Ligase is added. This would normally result in the generation of long linear concatemers of vector and insert DNA, but if (as depicted here) the vector is dephosphorylated and in large excess, the majority of products will be the vector::insert::vector trimolecular hybrids shown. The vector components will be in direct repeat (as shown LEFT) or in inverted repeated (as shown RIGHT) with equal probability. (Dephosphorylation also prevents regeneration of non-recombinant vector circles, which would result in this sytem from Cre recombination of vector::vector head-to-tail products.) (c) Stage2: After dilution of reaction to abolish macromolecular crowding, Cre is added. When the lox sites in hybrid DNA are directly repeated, Cre generates a circular recombinant plasmid and linear (vector) fragment. However, when the lox sites are inverted, Cre instead causes lox-flanked DNA to undergo rounds of unproductive inversion. DNA is then transformed or electroporated into E. coli in the usual way.

Figure 3. Alu-PCR amplification of inserts in recombinants derived by turbo cloning of Alu-PCR products of B2.13 DNA. Plasmid DNAs from six recombinants were amplified using Alu primer 614 (Materials and Methods) and aliquots run on a 1.8% agarose gel. Approximate insert sizes are (bp) 1100, 1290, 640, 2000, 290 and 860 (lanes 1-6). (The lower molecular weight bands are PCR artefacts caused by, e.g., mispriming.) Lane 7 contains six markers as in Fig. 1.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Improvements in or relating to cloning DNA

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGCACATAAC TTCGTATAAT GTATGCTATA CGAAGTTATG
40

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCGACATAAC TTCGTATAGC ATACATTATA CGAAGTTATG
40

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTGAGCCGAG ATCGCGCCAC TGCACT
26

Claims

1. A method of cloning a DNA molecule, comprising:

a) ligating a linear insert DNA molecule to linear vector DNA molecules, which vector molecules contain a single lox site, under conditions of macromolecular crowding so as to form at least some suitably ligated DNA in which a linear insert DNA molecule is joined at each end to a respective molecule of linear vector DNA wherein the two linear vector DNA molecules immediately adjacent to the insert DNA have the relationship of being direct repeats;

b) bringing the products of the ligation reaction, no longer under conditions of macromolecular crowding, into contact with Cre protein so as to cause circularisation of the suitably ligated DNA; and

c) dissociating the Cre protein from the DNA.

2. A method according to claim 1, wherein step b) is achieved intracellularly by introducing the DNA into a cell which is capable of expressing Cre.

3. A method according to claim 1 or 2, wherein step b) is achieved intracellularly by introducing the DNA into a cell which is capable of inducible expression of Cre.

4. A method according to claim 1, wherein step b) is achieved extracellularly and further comprising as step d); introducing the DNA into a suitable host.

5. A method according to claim 1 or 4, wherein the Cre

protein is dissociated from the DNA by heating.

6. A method according to any one of the preceding claims, wherein conditions of macromolecular crowding are achieved by the presence of 15% (w/v) polyethylene glycol 6000.

7. A method according to any one of the preceding claims, wherein the conditions of macromolecular crowding are removed by dilution of the reaction mixture.

8. A method according to any one of the preceding claims, wherein the vector molecule is p9lox5.

9. A method according to any one of the preceding claims, wherein the vector has been dephosphorylated prior to ligation.

10. A method according to any one of the preceding claims, wherein the linear insert DNA has blunt ends.

11. The plasmid p9lox5 as hereinbefore defined.

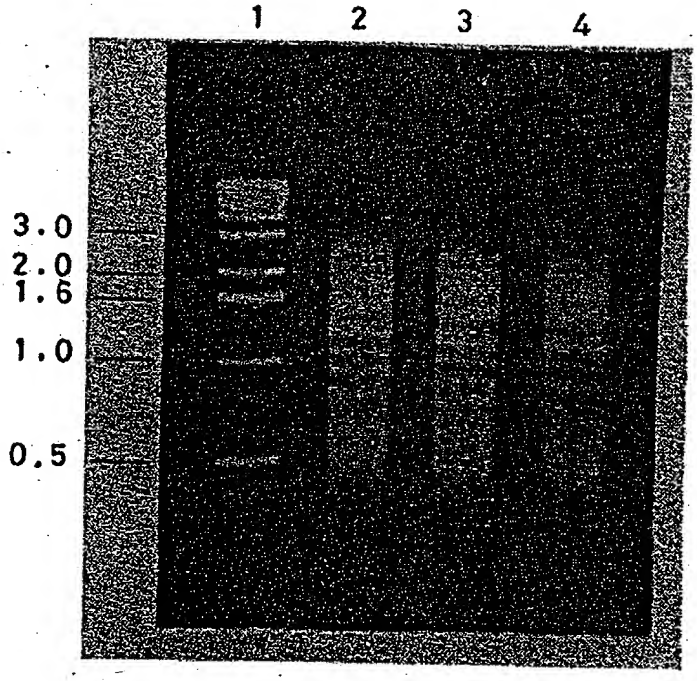



Fig. 1

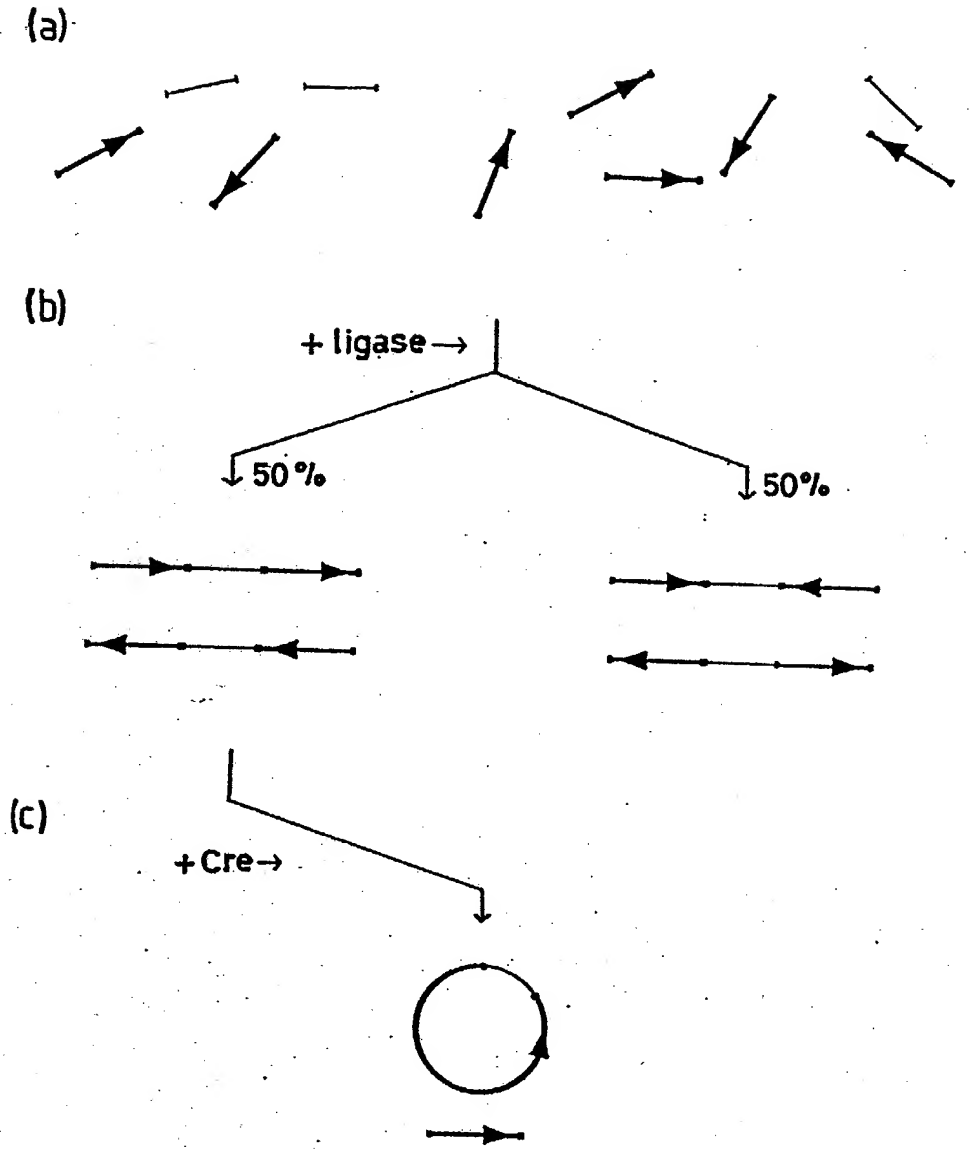
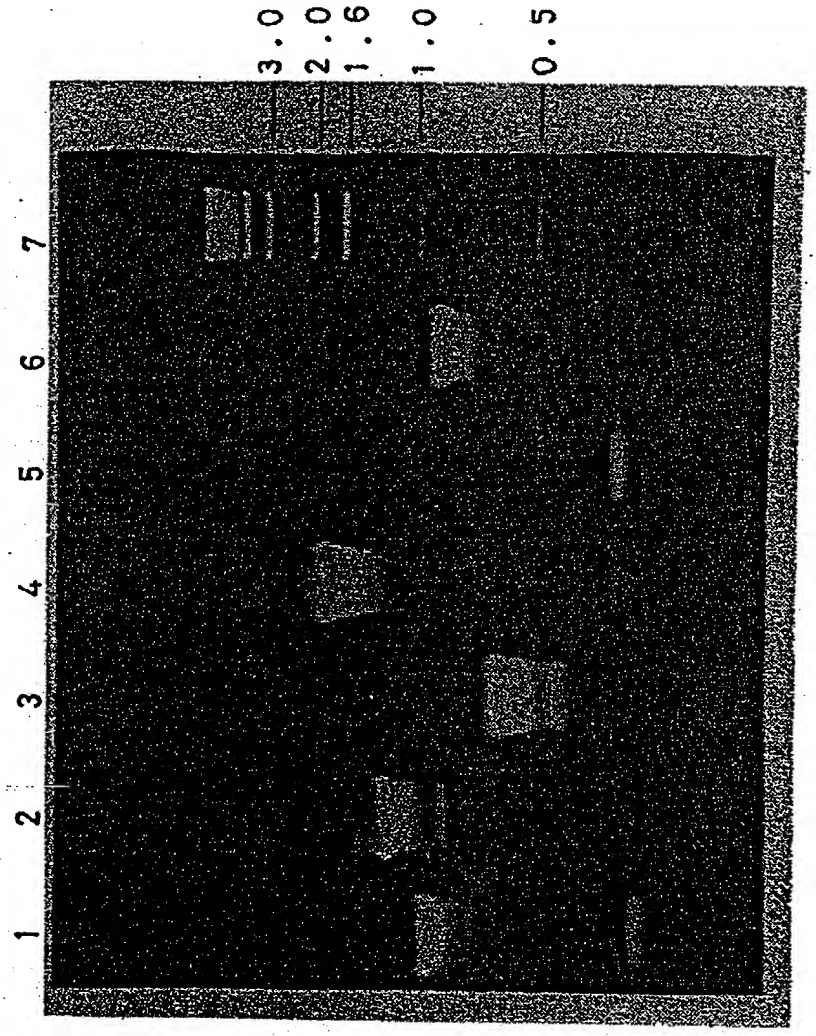



Fig. 2

Fig. 3



# INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 94/00272

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/66 C12N15/68 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | GENE.<br>vol. 51, no. 1 , 1987 , AMSTERDAM NL<br>pages 69 - 75<br>PETER UPCROFT ET AL. 'Rapid and efficient method for cloning of blunt-ended DNA fragments'<br>cited in the application<br>see abstract<br>see page 70, left column, paragraph 2<br>see page 71, left column, paragraph 2 - page 72, left column, paragraph 1<br>---<br>-/-- | 1,2 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 27 April 1994 | 28 -06- 1994 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | Montero Lopez, B |

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# INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 94/00272

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | GENE.<br>vol. 70, no. 2 , 1988 , AMSTERDAM NL<br>pages 331 - 341<br>BRIAN SAUER ET AL. 'The cyclization of linear DNA in Escherichia coli by site-specific recombination'<br>see abstract<br>see page 332, left column, paragraph 2 - right column, paragraph 1<br>see page 334, right column, paragraph 2<br>see page 336, left column, paragraph 2 - right column, paragraph 1<br>see page 340, left column, paragraph 1<br>see page 340, right column, paragraph 1 | 1,2 |
| A | EP,A,0 220 009 (E.I. DU PONT DE NEMOURS AND COMPANY) 29 April 1987<br>see page 2, line 33 - line 41<br>see page 3, line 25 - line 50<br>see page 4, line 1 - line 27 | 1-3 |
| P,X | NUCLEIC ACIDS RESEARCH.<br>vol. 21, no. 4 , 25 February 1993 , ARLINGTON, VIRGINIA US<br>pages 817 - 821<br>A. CHRISTOPHER BOYD 'Turbo cloning: a fast, efficient method for cloning PCR products and other blunt-ended DNA fragments into plasmids'<br>cited in the application<br>see the whole document | 1-11 |

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Int. ional Application No
PCT/GB 94/00272

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| EP-A-0220009 | 29-04-87 | CA-A- | 1293460 | 24-12-91 |
| | | DE-A- | 3687734 | 25-03-93 |
| | | JP-A- | 62175184 | 31-07-87 |
| | | US-A- | 4959317 | 25-09-90 |